ISOLATION AND CHARACTERIZATION OF CYANOGEN BROMIDE PEPTIDES OF RICE α-GLOBULIN AND PURIFICATION AND CHARACTERIZATION OF THE 12K CORN INHIBITOR

BY

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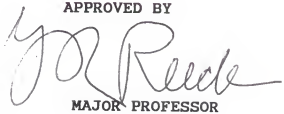

MAJOR PROFESSOR

DEDICATE TO MY MOTHER

AND

MY FATHER (IN MEMORY)

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TABLE OF CONTENTS

PART ONE: PEPTIDES OF RICE α-GLOBULIN

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I. LITERATURE REVIEW

A. INTRODUCTION

The storage proteins of seeds are an important group of plant proteins. They provide a nitrogen source during the germination of seeds and contribute a major protein source for human and livestock consumption. The study of storage proteins may provide information on improving their quality as well their quantities in cereals. The rather large quantities of these proteins make the study of their synthesis particularly attractive as models of genetic regulation (Larkins,1981).

Rice and wheat are the most important food cereals. Rice generally considered a semiaquatic, annual grass plant. Rice belongs to the same family as barley, oats, rye and wheat. These species, therefore, share many characteristics.

The edible part of rice, the caryopsis (brown rice), as shown in Fig. 1, consists of seed coat, nucellus, endosperm, and embryo (Juliano and Aldama, 1937). Outside

of the caryopsis is hull. The major part of the caryopsis is endosperm, which serves as a store of food reserves for the seed during germination. Endosperm consists of two parts, the outside subaleurone layer, and the central, starchy endosperm.

Protein is the second most abundant component in rice after starch. The protein of rice is deposited in protein bodies. Although rice has the lowest protein content among the cereals, it is relatively rich in lysine and better balanced in its amino acid composition than most other cereals (Juliano, 1985). Nevertheless, lysine is the first limiting amino acid in rice as in other cereals.

There is a large variation in protein content among rice cultivars. The protein content among the 17,587 cultivars of brown rice in the world collection at the International Rice Research Institute (IRRI) ranged from 4.3 to 18.2%, with a mean of 9.5% (Gomez,1979). Unfortunately, the protein content and the lysine content of milled rice are negatively correlated (IRRI, 1973).

B. PROTEIN FRACTIONS

Osborn (1907) differentiated protein into four fractions based on their solubilities :

albumin fraction ---- water-soluble proteins

globulin fraction ---- salt-soluble proteins

prolamin fraction ---- alcohol-soluble proteins

glutelin fraction ---- alkali/acid-soluble proteins.

The mass ratio of albumin, globulin, prolamin and glutelin in brown rice has been reported as 9:7:4:80 (Mitra and Das, 1975), 11:15:3:71 (Chavan and Duggal, 1978), 11:10:2:77 (Wieser et al., 1980). Padhye and Salunkhe (1979) reported 8:10:12:70 for them mass ratio of the four protein fractions of milled rice.

The proportion of albumin and globulin in rice is highest in the outer layers of the endosperm and it decreases toward the kernal center, whereas the proportion of glutelin has an inverse distribution (Houston et al., 1968). Radial distribution of amino acids in rice kernels has been studied by Hayakawa et al. (1987), whose results suggest that the nutritive value of rice protein may be reduced toward the middle layer and elevated slightly in the kernel center.

Analysis of milled rice fractions showed albumin has the highest lysine content, followed by globulin and glutelin. Prolamin has the lowest lysine content (Juliano and Boulter, 1976; Mandac and Juliano, 1978; Perdon and Juliano, 1978; Padhye and Salunkhe, 1979; Wieser et al., 1980). Globulin is richest in the sulfur-containing amino acids cysteine and methionine. Prolamin is the poorest.

C. PROTEIN BODIES

The rice proteins synthesized in the developing

endosperm are deposited in protein bodies. All of protein bodies are thought to initiate by dilation of the endoplasmic reticulum (Oparka and Harris, 1982). The transport of glutelin from its site of synthesis, the endoplasmic reticulum, to the site of deposition, the protein bodies, is mediated by the Golgi apparatus (Krishnan et al., 1986).

The protein bodies are not homogeneously distributed in rice endosperm. Bechtel and Pomeranz (1978) observed three types of protein bodies; large spherical, small spherical and crystalline protein bodies in the subaleurone region. The central region of endosperm lacked both small spherical and crystalline protein bodies. The central region appeared to be poor in lysine and had lower molecular weight polypeptides than the whole protein bodies (Tanaka et al., 1978).

Harris and Juliano (1977) examined two varieties of rice differing in protein content and found that high protein grain contained more protein bodies as well as rough endoplasmic reticulum. Increase in protein content was the result of increased number of protein bodies rather than increase in size.

Tanaka et al. (1980) observed two kinds of protein bodies in rice endosperm; spherical with lamella structure (PB-I). and one which stained homogeneously by osmium tetraoxide and lacked lamella structure (PB-II). Prolamin

was found in PB-I, while glutelin and globulin were found in PB-II.

Ogawa et al. (1987) isolated and purified PB-I and suggested that it contains several polypeptide groups, the largest of which has a molecular weight of 13 kDa. The minor polypeptides has molecular weight 10 and 16 kDa. Most of 13 kDa group were prolamins although it contains some salt and alcohol insoluble polypeptides. The protein contents in PB-I account for about 20% of total protein.

Sugimoto et al. (1986) suggested that glutelin in the PB-II was assembled from heterogeneous polypeptides, with molecular weights estimated to be 64, 140, 240, 320, 380, and 500 kDa. High molecular weight proteins (>2000 kDa) were also observed. SDS-PAGE under reducing conditions showed that glutelin in the protein bodies was composed of 2 polypeptide groups, 22-23 and 37-30 kDa, bound by disulfide linkage.

D. ALBUMIN

Albumin contains numerous components. Iwasaki et al. (1972) found 11 albumin bands of unpolished rice by starch gel electrophoresis at pH 3.1, and 15 bands at pH 8.9. Cagampang et al. (1976) resolved four major and six minor proteins by disc gel electrophoresis. SDS-gel electrophoresis showed three albumin bands with molecular weights of 11, 8.5 and 16 kDa. Pan (1984) observed

approximately 40 spots of rice albumin by two-dimensional gel. This method therefore resolved many more polypeptides than any other methods.

Extraction of milled rice of variety IR36 (Villareal and Juliano, 1981) showed that albumin solubilized by 0.1-0.15M ammonium sulfate consists of about 20% high (5%) lysine and 80% lower (2%) lysine protein. The 2%-lysine protein contains only one major fraction with a molecular weight of 17-19 kDa. The albumins exhibit three fractions by DEAE-Sephacel chromatography at pH 8.5. The three fractions exhibit different gel electrophoretic and isoelectric focusing patterns.

Mawal et al. (1987) isolated a major rice albumin which is a glycoprotein with a molecular weight of 60 kDa and pI of 6.54. Immunoassay showed that this albumin is not immunologically cross-reactive with rice globulin.

Albumin content progressively increases during grain development. Amino acid analysis showed that lysine content of albumin changes very little during rice development (Cagampang et al., 1976). The maximum albumin content is observed between 18 and 20 days post-anthesis (Mawal,1987).

E. GLOBULIN

Globulin can be classified into α, β, and γ fractions in order of the increasing rate of sedimentation in the

ultracentrifuge (Danielsson, 1949). Morita and Yoshida (1968) identified globulin by using ultracentrifugation and gel-filtration chromatography. γ-Globulin was found mainly in embryo and bran. On the other hand, α- and β-globulin were found to be more concentrated in the endosperm. The physical and chemical properties of γ-globulin have been extensively studied (Sawai and Morita, 1970a,b,c; Morita and Yoshida, 1968; Morita and Horikoshi, 1972; Morita et al. 1971).

The globulin in the endosperm appeared to have two major fractions with molecular weights 12 and 20 kDa (Shadi and Djurtoft, 1979, Cagampang et al., 1976, Houston and Mohammmad, 1970). The lower molecular weight globulin is more soluble and is higher in sulfur-containing amino acids (Houston et al., 1964). This fraction seemed to be composed of two groups of polypeptides which themselves are heterogeneous (Pan, 1984). The other fraction was classified as α-globulin based on its sedimentation coefficient.

Houston and Mohammad (1970) isolated α-globulin from a milled short grain rice endosperm. This protein represented 40% of the total globulin. The protein was extracted with 5% NaCl followed by ammonium sulfate and pH 4.5 precipitation. The protein has high glutamic acid and arginine, contains 1% tryptophan and essentially no lysine or histidine. Molecular weight determined by Sephadex G-

100 was about 25 kDa.

Perdon and Juliano (1978) suggested that a pH dependent aggregation of α-globulin can occur. The α-globulin exhibited one band by electrophoresis at pH 4.5 but two bands at pH 8.3. Gel filtration at pH 6.5 showed two proteins with molecular weights 20 and 98 kDa. The α-globulin also contained 8% carbohydrate. Pascual et al. (1981) suggested α-globulin has a major band with a pI 5.3 on isoelectric focusing gel.

Pan and Reeck (1988) developed an isolation procedure which avoids low pH conditions. The globulin fraction was separated from low molecular weight protein on a hydroxyapatite column at neutral pH. This procedure is most suitable for the study of this protein under native conditions. The molecular weight, determined by equilibrium centrifugation, was 16.7 kDa. The equilibrium centrifugation method should give a more accurate estimate of molecular weight than other methods. The extinction coefficient (0.1%, 280 nm) was determined as 1.07.

F. GLUTELIN

Glutelin is the major storage protein in rice. It contains two major polypeptide groups: one with molecular weight of 20-25 kDa, the other with molecular weight 35-39 kDa (Villareal and Juliano, 1978; Juliano and Boulter, 1976; Krishnan and Okita, 1986; Robert et al., 1985; Wen

and Luthe, 1985; Zhao et al.,1983; Yamagata et al., 1982). A third one with molecular weight 14-16 kDa (Juliano and Boulter,1976; Krishnan and Okita, 1986) was suggested to be a contaminating prolamin (Tanaka et al., 1980; Krishnan and Okita, 1986).

Electrofocusing (Wen and Luthe, 1985) indicated that the 20-25 kDa group is basic (pI 9.4-10.3) and the 35-39 kDa group is acidic (pI 6.5-7.5). Both groups are highly heterogeneous.

Glutelin has been shown to be derived from a larger precursor (Krishnan and Okita, 1986; Furuta, et al., 1986; Sarker et al., 1986). Rice glutelin is suggested to be a protein homologs to pea legumin (Zhao et al. 1983), or oat globulin (Robert et al., 1985). Nucleotide sequences of DNA molecules also confirmed that rice glutelin is a glycinin- or legumin-like protein (Takaiwa et al., 1986; Takaiwa et al., 1987a,b; Wang et al., 1987, Higuchi and Fukazawa, 1987). Rice glutelin gene clones could be divided into two groups based on their restriction site pattern (Takaiwa, et al., 1987b) and difference in 3'-noncoding region (Wang, et al., 1987). Southern blot hybridization suggested that there are four or five copies of glutelin gene per haploid rice genome (Takaiwa, et al., 1987b).

G. PROLAMIN

Unlike other cereals, prolamin is a minor fraction of rice endosperm. Early work by Tecson et al. (1971) suggested that preparations of prolamin may be contaminated by polyphenol. Mandac and Juliano (1978) developed a lipid- and phenol-free prolamin from milled IR480-5-9. The preparation consisted mainly of one band on SDS-polyacrylamide gel electrophoresis with a molecular weight 17 kDa and a minor fraction with 23 kDa. Tanaka et al. (1980) reported the major fraction with molecular weight 13 kDa. Rice prolamin is the fraction poorest in lysine, histidine, cystine, methionine but rich in glutamic acid, tyrosine and proline. Isoelectric focusing showed that the pI of prolamin ranged from 6.0 to 6.5 (Padhye and Salunkhe, 1979). Cell-free synthesis of rice prolamin suggested that prolamin was synthesized with a signal peptide (Yamagata et al., 1986).

H. RICE α-GLOBULIN AS A TARGET FOR PROTEIN ENGINEERING

To many people in developing countries, rice provides not only a source of calories, but also serve as nitrogen source containing essential amino acids. Rice, like other cereals, is not balanced in essential amino acids. The first limiting amino acid is lysine (FAO/WHO, 1973).

One reasonable method in increasing rice lysine content is substituting lysine for arginine residues.

Lysine-for-arginine is one of the most common interchange in the evolution of homologous proteins (Dayhoff, et al., 1972). It preserves positive charges, minimizing the disruption of three-dimensional structure.

With the advent of recombinant DNA technology and ancillary DNA sequencing techniques, we now can study gene expression at molecular level. It is now possible to create mutations at predetermined sites on DNA molecules.

Rice α-globulin is the major protein in the globulin fraction. It contains about 12% arginine and contains essentially no lysine, as shown in Table 1 (Pascual et al., 1981; Houston and Mohammad, 1970; Perdon and Juliano, 1978). From a nutritional point of view, lysine-for-arginine interchanges will increase the first limiting amino acid of rice at the expense of a non-essential amino acid. If this kind of substitution succeeds, we can improve the nutritional balance of amino acids in rice.

I. SPECIFIC AIMS ON THIS WORK

In order to study site-specific mutagenesis of α-globulin, we need to isolate a full length cDNA of α-globulin. Since there is not any information about the structure of this protein, we need to obtain partial sequences of it. From these partial sequences we design a synthetic DNA probe to isolate its cDNA. This partial sequence can also be used to verify the cDNA sequence.

In this study I used cyanogen bromide to digest rice α-globulin. Digested peptides were purified and subjected to automated Edman degradation to determine portions of the protein's amino acid sequence.

II. MATERIALS

A. PLANT MATERIALS

Rice (*Oryza sativa*, Newbonnet cultivar) was generously provided by Dr. Robert Dilday of the USDA Rice Center, Stuttgart, Arkansas. Rice was milled, ground to 68 mesh, defatted with four volumes of acetone for one hour, and air-dried under a fume hood overnight.

B. REAGENTS

Unless specified, all chemicals are reagent grade or better, obtained mostly from Fisher or Sigma.

The following reagents were used in HPLC:

Trifluoroacetic acid -- HPLC/Spectro grade; Pierce

Heptafluorobutyric acid -- HPLC grade; Pierce

Acetonitrile --HPLC or Optima grade; Fisher

Water -- HPLC bottle water (Fisher) or from Milli-Q water system (Waters)

Triethylamine -- HPLC grade; Pierce

Sodium acetate -- HPLC grade ; Fisher

Guanidine HCl -- 8 M solution, Sequanal grade; Pierce

Phenylisothiocyanate -- 1 ml ampules, Sequanal;

Pierce

Hydrochloric acid -- 1 ml ampules, Sequanal grade; Pierce

Acetic acid -- HPLC grade; Baker

C. HPLC COLUMNS

Synchropac RP-P (250x4.1 mm)-- Synchrome

Pico-Tag amino acid analysis column (150x3.9 mm) --Waters

TSK 2000SW (600 x 7 mm) -- Varian

III. METHODS

A. PURIFICATION OF α-GLOBULIN

Purification was carried out as described by Houston and Mohammad (1970). Defatted rice flour was extracted with 4 volumes of 0.8 M NaCl for 2 hours at room temperature. Solids were removed by centrifugation. The filtrate was brought to 30% saturation of ammonium sulfate to form a precipitate. The precipitate was centrifuged, redissolved in 0.4 M NaCl, The protein was again precipitated with 30% ammonium sulfate. The precipitate was dissolved in 0.4 M NaCl and dialyzed against deionized water overnight (two changes of water) and lyophilized. The resulting fraction was termed the globulin fraction.

The globulin fraction was dissolved in 1% acetic acid. NaOH (0.5 M) was added to bring the pH to 4.5 to form a precipitate. The precipitate was centrifuged and redissolved in 1% acetic acid. The pH 4.5 precipitation was performed three times. Finally the precipitate was dissolved in 1% acetic acid, dialyzed against water, and lyophilized.

B. REDUCTION AND *S*-PYRIDYLETHYLATION

Ten mg of protein was dissolved in 6 M guanidine hydrochloride containing 0.13 M Tris-HCl and 0.3 mM EDTA, pH 7.6. After addition of 1.5 mg dithiothreitol, the

solution was incubated at room temperature for 22 hr. Then 3 µl of 4-vinylpyridine was added and the mixture was allowed to stand for 90 min. The reaction mixture was then desalted on a 600 x 7 mm TSK-2000SW column using 0.15% ammonium-trifluoroacetic acid/20% acetonitrile/80% H_2O, pH 3.2, as solvent.

C. CYANOGEN BROMIDE CLEAVAGE

Forty mg of cyanogen bromide (Aldrich) was dissolved in 70% formic acid and immediately added to 10 mg of reduced and *S*-pyridylethylated α-globulin (5 mg/ml). After 24 hr, 20 ml of water was added to the reaction mixture, which was then lyophilized (Nute and Mahoney,1979).

D. HPLC SEPARATION OF PEPTIDES

The lyophilized CNBr digest was dissolved in 0.1% trifluoroacetic acid or 0.1% heptafluorobutyric acid containing 2 M guanidine hydrochloride and subjected to reversed-phase HPLC on a Synchropak RP-P column (250 x 4.1 mm) coupled with a guard column (50 x 4.1 cm). The column was equilibrated with solvent A (either 0.1% trifluoroacetic acid or 0.1% heptafluorobutyric acid in water). The peptides were then eluted using a gradient of acetonitrile in either 0.1% trifluoroacetic acid or heptafluorobutyric acid. The flow rate was 1 ml/min. The absorbance of the effluent was monitored at 214 nm.

Individual peaks were collected and lyophilized.

Gel permeation high performance liquid chromatography was performed on a TSK 2000SW (600 x 7 mm) column coupled with a guard column (75 x 7 mm). The column was equilibrated with 6 M urea containing 0.1 M NaH_2PO_4, pH 5.5. The sample was dissolved in the same buffer and injected into the column. The flow rate was 0.5 ml/min and the absorbance of the effluent was monitored at 280 nm. Individual fractions were collected, dialyzed and lyophilized.

E. AMINO ACID ANALYSIS

Samples were dried in 0.6 cm x 5 cm borosilicate glass tubes, evacuated and hydrolyzed with 6 M HCl at 110°C for 22-24 hours in a Waters amino acid hydrolysis vial. The hydrolysates were dried, redried and derivatized with phenylisothiocyanate (Bidlingmeyer et al., 1984). A standard amino acid mixture was dried, redried and derivatized in the same way.

The derivatized sample was dissolved in 50-200 ul Waters Pico Tag sample diluent and analyzed by a Pico Tag (Waters) amino acid analysis column at 40°C. Results were reported as average of 2-4 analysis.

F. AMINO ACID SEQUENCING

Automated Edman degradation was performed on peptides

with a Beckman Sequencer as previously described (Mahoney et al. 1984) by Dr. Mark Hermodson at Purdue University.

IV. RESULTS

A. CYANOGEN BROMIDE DIGESTION

Reduced and *S*-pyridylethylated α-globulin was digested with cyanogen bromide. SDS gel electrophoresis (not shown) demonstrated that the digest contained a rather large fragment with an apparent molecular weight of about 10kDa and several low molecular weight peptides, which did not resolve well from each other. The existence of a large peptide suggested that a rather long stretch of α-globulin contained no methionine.

B. PEPTIDES PURIFICATION

Reversed-phase HPLC was used to separate cyanogen bromide peptides. In the initial separation, 0.1% trifluoroacetic acid was used as an ion pairing reagent. The main peaks (Fig. 2) were collected and lyophilized. An unresolved region (RP-2/RP-3) was further purified by chromatography on the same column but with 0.1% heptafluorobutyric acid (Fig. 3, Fig. 4). Peak RP-4 (Fig. 2) contained a large fragment in an impure state (not shown). The recovery of this fragment was low. The large fragment was purified by high performance gel permeation chromatography (Fig. 5) and named GPC-1.

C. AMINO ACID ANALYSIS OF PEPTIDES

Amino acid analysis (Table 2) suggested that RP-2 was the C-terminal peptide because it contained no homoserine. RP-1 and RP-3 were rather simple in composition, which is consistent with their apparently low molecular weights. GPC-1 contained unusually high amounts of glutamic acid/glutamine, serine, glycine and tyrosine.

D. AMINO ACID SEQUENCES

The amino acid sequences of RP-1 and RP-2 ,and the partial amino acid sequence of the GPC-1 peptide were determined by automated Edman degradation (Table 3). RP-3 was evidently blocked since no phenylthiohydantoin-amino acid was released from it. This suggests that RP-3 is the N-terminal peptide because, in trying to sequence α-globulin itself, we found its N-terminal was also blocked (Pan and Hermodson, personal communication). The partial sequence of GPC-1 showed a peculiar arrangement in which glutamic acid, serine and tyrosine appeared in groups of up to 5 amino acids. The partial amino acid sequence of GPC-1 showed heterogeneity at several positions.

V. DISCUSSION

In this study, trifluoroacetic acid was used in the first dimension and heptafluorobutyric acid in the second dimension in reversed-phase HPLC to purify protein fragment RP-2 and RP-3. This procedure gives some advantages over using other ion pair reagents. Trifluoroacetic acid is highly UV-transparent, volatile, and soluble in common eluent. Hepataflurobutyric acid affects the retention of peptides differently than does trifluoroacetic acid. This method allows two dimensional purification of peptides with a single column. Bennett et al. (1980) routinely used these two reagents in the final purification of several peptide hormones. Bishop et al. (1980) also used them in preparative purification of peptides. The method results in good separation of peptides in this application.

This study reports the first amino acid sequence information for α-globulin from rice endosperm. The 4 cyanogen bromides peptides that we were isolated apparently represent much of the protein, since the sum of the molecular weight of these four peptides is close to the molecular weight of intact α-globulin. This protein was suggested to contains 3.4-4.6 mole% methionine (Pascual et al., 1981; Houston and Mohammad,1970, and Perdon and Juliano, 1978) and thus should be cleaved into

more than 4 peptides. The existence of a large peptide (GPC-1) suggests that if any other peptides are left, they should be small. A diagram shows the proposed position of these four peptides generate by this study (Fig. 6).

This study of the 4 peptides reveals a far-from-uniform distribution of certain amino acids within the protein. The 3 short peptides appear to have a mixture of hydrophilic and hydrophobic residues that is at least roughly characteristic of folded domains in proteins. The large cyanogen bromide peptide (GPC-1) has a most peculiar composition in that it consists, in large part, of three amino acids: (Glx, Ser, and Gly). That portion of the sequence of GPC-1 that we determined shows that individual, abundant amino acids tend to occur in short stretches consisting of one amino acid, for instance Ser, Tyr. Such stretch will probably not form α-helix since Ser, Tyr and Gly are not helix formers (Chau and Fasman, 1978). The α-helix in α-globulin was determined as 49% (Pan and Reeck, 1988).

The amino acid sequence of α-globulin in the large fragment of peptide suggests that this protein exhibit heterogeneity. This result is confirmed by the two-dimensional electrophoresis analysis of salt extracted protein (Pan, 1984), which showed that one major spot of α-globulin and a faint spot near major spot at basic side.

S.-J. Pan (personal communication) of this laboratory

used the amino acid sequence information obtained in this study to synthesize oligonucleotide probes to allow the isolation of cDNA clones coding for α-globulin. This method was not succeed. This may due to considerable degeneracy of the synthesized oligonucleotides sequence. Recently B. Shorrosh et al. (personal communication, 1988) in this laboratory has isolated a cDNA clone encoding α-globulin by screening an expression library with antibodies against α-globulin. The amino acid sequence inferred from the cDNA contained the sequence of RP-2 and GPC-1. The complete sequence of α-globulin will be deduced and site specific mutagenesis will be studied in the future.

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Fig. 1. Structure of rice grain (adapted from Juliano and Aldama, 1937).

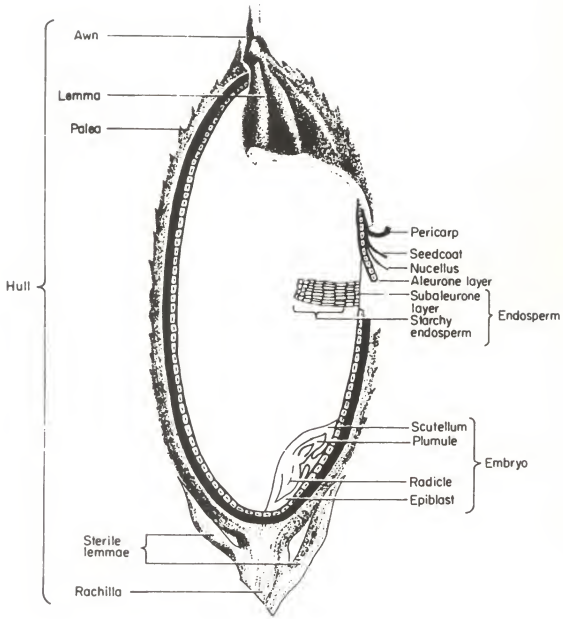
Awn
Lemma
Palea
Hull
Pericarp
Seedcoat
Nucellus
Aleurone layer
Subaleurone layer
Starchy endosperm
Endosperm
Scutellum
Plumule
Radicle
Epiblast
Embryo
Sterile lemmae
Rachilla

Fig. 2. Separation of CNBr peptides of rice α-globulin by reversed-phase HPLC using 0.1% TFA as an ion pairing reagent. The column was equilibrated with 0.1% TFA in water and developed with an acetonitrile/0.1% TFA gradient (0-12%B in 30 min, 12-24%B in 90 min).

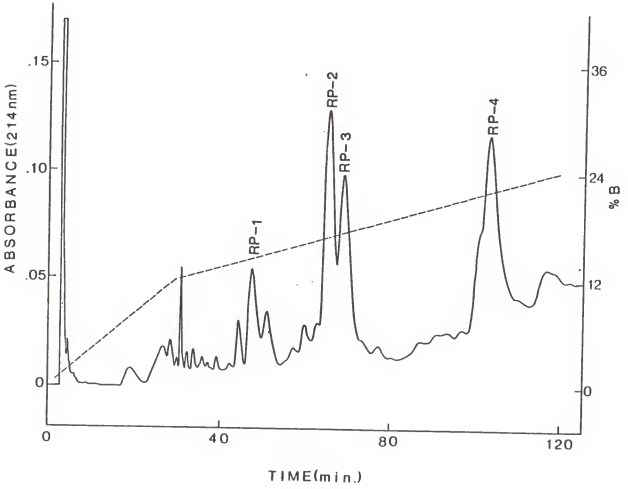
ABSORBANCE(214nm)
.15
.10
.05
0
RP-1
RP-2
RP-3
RP-4
36
24
12
0
% B
0
40
80
120
TIME(min.)

Fig. 3 Reversed-phase HPLC purification of peak RP-2 from Fig. 2 using 0.1% heptafluorobutyric acid (HFBA) as an ion pairing reagent. Solvent A: 0.1% HFBA in water. Solvent B: 0.1% HFBA in acetonitrile. The column was equilibrated with 20% solvent B and developed in a linear gradient to 40% solvent B in 60 min.

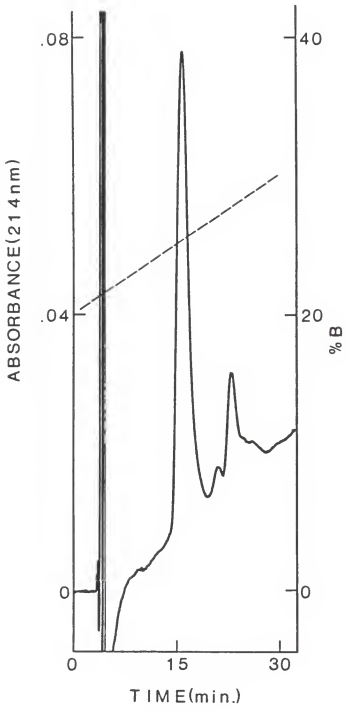
.08
.04
0
40
20
0
%B
ABSORBANCE(214nm)
0
15
30
TIME(min.)

Fig. 4. Reversed-phase HPLC purification of peak RP-3 from Fig 2. Condition used was the same as in Fig. 3.

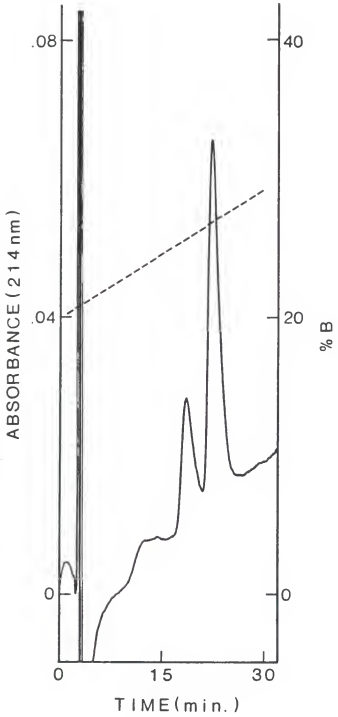
.08
.04
0
ABSORBANCE (214 nm)
40
20
0
%B
0
15
30
TIME (min.)

Fig. 5 Gel permeation HPLC separation of the CNBr peptides of rice α-globulin. The solvent consisted of 6 M urea and 0.1 M NaH_2PO_4. Inset: Rechromatography of peak GPC-1 under the same conditions. Material from the early part of the GPC-1 peak (indicated by bar) was applied to the same column and chromatographed under the same conditions.

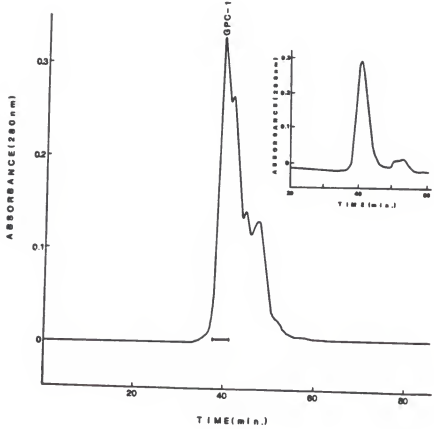
GPC-1
ABSORBANCE(280nm)
0.3
0.2
0.1
0
20
40
60
80
TIME(min.)

Fig. 6. The relationship of rice α-globulin CNBr fragments to the entire sequence. The first solid line represent the whole sequence of α-globulin. Its molecular weight was estimated by Pan and Reeck (1988). Solid lines in peptides represent known sequence, dashed lines represent unknown sequence. The parentheses represent the fact that the relative position of these two sequence in intact protein is unknown.

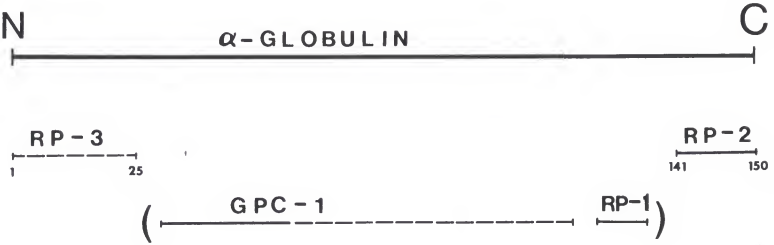
N
α-GLOBULIN
C
RP-3
1
25
RP-2
141
150
GPC-1
RP-1

Table 1 Amino acid composition of rice α-globulin[1,2]

	A	B	C
Asp	3.3	4.7	3.8
Glu	23	24	25
Ser	11	11	10
Gly	8.6	8.5	8.5
His	trace	0.3	0.1
Arg	14	11	14
Thr	1.9	2.3	2.0
Ala	5.6	5.0	5.7
Pro	4.9	5.4	5.1
Tyr	5.4	4.7	5.5
Val	3.8	4.4	3.5
Met	4.6	3.4	3.6
Cys	4.5	3.1	3.4
Ile	1.3	1.8	1.3
Leu	6.1	6.3	6.2
Phe	2.4	2.6	2.6
Lys	0.1	0.9	0.5

1. A, B, C, are the amino acid composition of α-globulin reported by Houston and Mohammad (1970), Perdon and Juliano (1978), and Pascual et al (1981), respectively.

2. Mole per cent.

Table 2 Amino acid compositions of CNBr peptides from rice α-globulin[1,2]

	RP-1	RP-2	RP-3	GPC-1
Asp	0.00	0.00	4.56	0.96
Glu	2.05	23.82 (3)	21.55	22.12
Ser	0.00	5.36 (1)	4.81	12.18
Gly	16.08 (2)	6.19 (1)	3.25	19.17
His	0.00	0.00	0.00	0.00
Arg	33.10 (2)	8.06 (1)	22.11	8.56
Thr	0.00	0.00	0.00	3.05
Ala	9.73 (1)	13.27 (2)	9.79	5.16
Pro	8.79 (1)	6.46 (1)	0.00	6.43
Tyr	0.00	5.33 (1)	0.00	7.04
Val	0.00	6.15 (1)	4.40	3.06
Met[3]	5.00 (1)	0.00	1.86	0.85
Ile+Cys[4]	0.00	18.79 (1+2)	20.73	1.51
Leu	15.47 (2)	0.00	5.10	5.75
Phe	10.05 (1)	6.48 (1)	2.46	3.63
Lys	0.00	0.00	0.00	0.53

1. Mole percent.
2. Data obtained from sequence (see Table 2).
3. Determined as homoserine.
4. The *S*-pyridylethyl cysteine and isoleucine phenylthiohydantoin derivatives were not resolved. Therefore the contents of these two amino acids are reported together.

Table 3 Amino acid sequence of CNBr peptides from α-globulin

Peptide	Sequence
RP-1	FRRPGALGLM[1]
RP-2	CRVEPQQCSIFAAGQY[2]
GPC-1 (partial)	GLEQEWSSSSSEYYYGEGSSSEQ?YY[3]

1. Determined as homoserine.
2. Trace leucine appeared at position 5.
3. The following amino acid also appeared: position 1 (P,S), 3 (S), 4 (Y,V), 5 (G), 15 (G).

Part TWO: PURIFICATION OF THE 12K CORN INHIBITOR

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I.LITERATURE REVIEW

A. INTRODUCTION

Protease inhibitors are ubiquitously distributed in plants. Although the most intensely studied plant protease inhibitors are from Leguminosae (beans and peas), the study of protease inhibitors from cereals has increased during the last decade. A brief review on cereal protease inhibitors can be found in Boisen (1983).

The highest activity of protease inhibitors is in seeds. It has been suggested that the biological functions of protease inhibitors are both regulatory and protective (Ryan, 1981), however most of the specific target enzymes are not known (Laskowski and Kato, 1980).

There are four classes of proteases: thiols, carboxy, metallo, and serine proteases. Each class has protein inhibitors, but the number of inhibitors of serine proteases far exceeds that of three other classes. Serine protease inhibitors can be divided into several families by sequence similarities. Most of serine protease inhibitors interact with proteases following the

"standard mechanism" as described by Laskowski and Kato (1980). Inhibitors following this mechanism are highly selective substrates that undergo limited proteolysis by their target enzymes. The specifically hydrolyzed peptide is the scissile bond at the reactive site. At neutral pH, the equilibrium constant between modified inhibitor (reactive site bond hydrolyzed) and virgin inhibitor (reactive site peptide bond intact) is near unity (Laskowsky and Kato, 1980).

Many cereal protease inhibitors are quite stable molecules and can resist heat and extreme pH. The stability may be attributed to the high content of cysteine which stabilizes the structure (Boisen, 1983). No plant inhibitor has been identified as a glycoprotein (Ryan, 1981).

B. CORN TRYPSIN INHIBITOR

Corn trypsin inhibitor is found mostly in the endosperm rather than in embryo (Halim et al., 1973). The genotype of the grain under study also affects the level of inhibitor. Varieties containing the opaque-2 gene exhibit much higher concentration than does floury-2).

A corn trypsin inhibitor was isolated by Hochstrasser et al. (1967). It was claimed to consist of two peptide chains connected by disulfide bridges. The molecular weight was determined to be about 20 kDa. The amino acid

sequence deduced led to the conclusion that the inhibitor is a trimer with 65 amino acid residues on each monomer (Hochstrasser et al., 1970). No tryptophan was found in this inhibitor.

Swartz et al. (1977) isolated a trypsin inhibitor from opaque-2 corn seeds by trypsin-agarose affinity chromatography. The isolated trypsin inhibitor was a mixture of modified (two-chain) and virgin (single-chain) forms. The virgin form had a molecular weight of approximately 12.5 kDa. In contrast to the result of Hochstrasser et al. (1970), this inhibitor contained several tryptophan residues. The primary and secondary structure of this inhibitor has been determined (Mahoney et al., 1984). The sequence has a peculiar Pro-Arg-Pro-Arg-Leu-Pro arrangement at the reactive site. This inhibitor combined with trypsin at a 1:1 stoichiometry Circular dichroism measurements suggested that this inhibitor has helix and β-structure contents of approximately 40 and 20%, respectively. There is little similarity between this sequence and that of Hochstrssser et al.(1970). One interesting property of Mahoney et al's(1984) inhibitor is that it is also a specific inhibitor of Hageman factor, an important blood coagulation enzyme (Hojima et al., 1980).

Richardson (1987) isolated a 22k bifunctional bovine trypsin/beetle α-amylase inhibitor from corn seeds. The

protein has no apparent sequence similarity to any other enzyme inhibitors but is similar in sequence to an intensively sweet protein thaumatin and to a protein induced in tobacco following infection by tobacco mosaic virus.

Abe (1980) isolated two thiol-protease inhibitors from endosperm of corn. The purified inhibitors have molecular weight of about 9.5 and 13 kDa.

Recently, Wen et al. (1988, personal communication) isolated a full length cDNA clone for corn 12k inhibitor. The sequence is quite similar to that of Mahoney et al. (1984), but the reactive site the protein has just one Pro-Arg pair and the sequence has seventeen more residues at the C-terminal with a tryptophan residue among them. The alignment of these two sequences are shown in Fig. 7.

The results above reveal that corn contains several different types of inhibitors. Within one type there may be heterogeneity. This is clearly the case for the 12k inhibitor (Hojima et al., 1980; Lei and Reeck, 1986). Amino acid sequence analysis has showen that heterogeneity exist in the 12k inhibitor from opaque-2 corn (Mahoney et al. 1984).

C. SPECIFIC AIMS OF THIS WORK

In this study I refined a reversed-phase HPLC method to give a more rapid method to purify corn 12k inhibitor

(Hageman factor inhibitor) in its single chain form from opaque-2 corn. I used two-dimensional gel electrophoresis and Western blotting to study its heterogeneity. These studies will allow a further examination of possible functional differences in the various forms of the corn inhibitor.

II. MATERIALS

A. Plant Materials

Corn (opaque-2) was generously given by professor Clyde A. Wassom. Corn was ground to pass through 140 mesh screen, defatted with acetone and air dried as in Part One of this thesis.

B. Reagents

Unless specified, the chemicals used were the same as in Part One.

Antisera against corn 12k inhibitor were prepared in this lab by Dr. M. Lei. The inhibitor was purified by passing through trypsin-agarose column twice (Lei and Reeck, 1986). The purified antigen was dissolved in saline and inject into two rabbits. Serum was collected after the rabbits were boosted twice. The specificity of the antibody was tested by immunoprecipitation.

The following reagents were used in isoelectrofocusing:

Urea -- electrophoresis grade; Bio-Rad

Acrylamide -- electrophoresis grade; Bio-Rad

Ampholine -- pH 3.5-10, pH 4-6, pH 5-7; LKB

C. HPLC COLUMN

Synchroprep-RPP (250 x 10 mm) : Synchrome

III. METHODS

A. CORN EXTRACT

Defatted corn flour was extracted with 2 volumes of 0.2M NaCl at 4°C overnight, and centrifuged at 12000xg. The supernatant was boiled in a water bath for 15 minutes and centrifuged. The supernatant was adjusted to pH 2.2, centrifuged and filtered through a 0.2µm nylon 66 filter.

B. TWO-DIMENSIONAL GEL ELECTROPHORESIS

Two-dimensional gel electrophoresis was performed by O'Farrell's method (1975). Isoelectric focusing was used as the first dimension and SDS-slab gel electrophoresis as the second dimension.

For the first dimension, the gel was composed of 9.2 M urea, 2 % nonidet P-40, and 2% ampholine. The upper (cathode) solution was 0.01M NaOH, the lower (anode) solution was 0.02 M H_3PO_4. The voltage was increased up to 800 volts in 1 hour and kept at that condition for 22 hours. The gel was then removed and equilibrated in equilibration buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 0.0625M Tris-HCl, 0.02% bromophenol, pH6.8).

Separation in second dimension was performed using 15% acrylamide/0.2% bis-acrylamide. The gel was run at 5-7 mA until the dye front reached the bottom. The gel was then stained with Coomassie blue R-250, and destained with 10%

acetic acid/20%ethanol.

C. WESTERN BLOTTING

Protein was either directly spotted onto nitrocellulose paper or separated by gel electrophoresis (one dimensional or two dimensional) then electroblotted onto nitrocellulose membrane. The membrane was air dried, soaked in 3% BSA /TBS solution (10 mM Tris, 140 mM NaCl, pH 8.0) for at least 30 min. The membrane was rinsed with TBS solution, then incubated in antisera against corn trypsin inhibitor which was diluted 1:100 with TBST (TBS solution with 0.05% Tween-20) or preimmune sera for 4 hours. The membrane was then washed with TBST 5 minutes for five times, then incubated with second antibody (Goat anti-rabbit IgG) for two hours. The membrane was washed thoroughly as before, then developed with HRP color reagent (20ml HRP color reagent, Bio-Rad/100ml TBS/60µl H_2O_2).

IV. RESULTS

A. REVERSED-PHASE HPLC PURIFICATION OF THE 12K CORN INHIBITOR

Lei and Reeck (1986) combined affinity chromatography and reversed-phase HPLC to purify the 12k corn inhibitor. The affinity trypsin-agarose column modifies the corn inhibitor into the two-chain form. Reversed-phase HPLC may provide a single step to purify corn trypsin inhibitor in virgin form. In this study a reversed-phase HPLC semi-preparative column was used to purify corn trypsin inhibitor in a single step.

In order to establish a new purification method, it first need to be established which fraction of the reversed-phase chromatogram contains the inhibitor. The acidified, boiled extract was applied to the semi-preparative column (250x10 mm), which was eluted with a linear gradient of acetonitrile from 0% to 60% over 60 min. The elution profile was shown in Fig. 8. Fractions were collected every two minutes, lyophilized and dissolved in TBS buffer. Aliquots were taken from each fraction and spotted onto nitrocellulose membrane. Antibodies against corn trypsin inhibitor were used to detect which fractions contained immunologically related proteins (Fig. 8, inset). The reaction was strong only at peak A in Fig. 8.

The fractions near peak A were further analyzed by SDS-PAGE and Western blotting (Fig. 9, lane 1-6). The results showed a strongly reactive band that comigrated with corn trypsin inhibitor (band A), and a few weakly reactive bands with higher molecular weights (band B, C). A similar Western blot pattern was obtained using corn trypsin inhibitor purified by trypsin-agarose column and reversed-phase HPLC (Fig. 9, lane 7).

In order to increase the yield of corn trypsin inhibitor obtained in a single run, the column was equilibrated to 30% acetonitrile before. Boiled extract (adjusted to 30% acetonitrile also) was injected into column. After the flowthrough peak emerged, the acetonitrile was increased to 60% in a linear gradient. A sharp peak which contained corn trypsin inhibitor was identified (Fig. 10). The amount of inhibitor in the peak was determined as 0.55 mg based on extinction coefficient 2.0 (0.1%, 280 nm). At least up to 50 ml of 30% diluted extract can be injected in a single run without any change of the shape of the inhibitor peak (Fig 11).

B. TWO DIMENSIONAL GEL ELECTROPHORESIS OF THE 12K CORN INHIBITOR

In order to better study the heterogeneity within the 12k corn inhibitor, the inhibitor peak was collected, lyophilized and analyzed by two-dimensional gel

electrophoresis. It showed that the inhibitor possesses heterogeneity. At least one major spot and three minor spots can be visualized (Fig. 12). These proteins have acidic pI's as shown on the gel. These spots were best separated using ampholyte of the pH range 4-7 (Fig. 13).

The two-dimensional gel was also electroblotted onto nitrocellulose paper and analyzed by immunoassay (Fig. 14). The result showed that the antibodies can recognize several proteins. The largest group has the same molecular weight as corn trypsin inhibitor, several more spots can also be seen. One group has higher molecular weight than corn trypsin inhibitor. Two spots of lower molecular weight also appeared.

V. DISCUSSION

The 12k corn inhibitor has been purified by various methods (Hojima et al., 1980, Swartz et al. 1977, Lei and Reeck, 1986, Chen and Mitchell, 1973). Conventional chromatography methods are tedious because they requires several steps to purify the inhibitor, and buffers needed to be changed between steps. Although affinity chromatography can give a high degree of purification, it can modify a portion of inhibitor into split-chain form. Another chromatography step is usually required to separate split-chain from virgin form. The new method report above is fast, reliable and will not modify the inhibitor into its split-chain form. Also it does not require any desalting steps since the solvents are totally volatile.

There is some evidence that the 12k corn inhibitor exhibits heterogeneity. Hojima et al. (1980) showed three fractions of corn trypsin inhibitor of sweet corn by preparative isoelectric focusing. Mahoney et al. (1984) obtained amino acid sequence of corn trypsin inhibitor and found that exists heterogeneity at position 91(Ala or Glu). Lei and Reeck (1986) combined trypsin-agarose and reversed-phase HPLC to purify corn trypsin inhibitor. The results also showed that heterogeneity may exist since reversed-phase HPLC resolved several peaks with similar

molecular weight. The Two-dimensional gel results above clearly showed that heterogeneity exists. At least four spots can be seen in the two-dimensional gel and numerous spots were seen in Western blot.

The Western blot of SDS-PAGE in Fig. 9 showed that at least three bands varying in size were recognized by antibodies. The result suggests size heterogeneity of the inhibitor. Interestingly, the cDNA clone isolated by Wen et al. (personal communication, 1988) is 15 amino acids longer than the corn trypsin inhibitor (Mahoney et al. 1984), which may correspond to a slightly higher molecular weight of protein on the Western blot (Fig. 9, band B).

The Western blot showed that spots of both higher and lower in molecular weight than corn trypsin inhibitor react with antibodies. These spots may be due to a contamination of the antigen. Alternatively this result may indicate that the higher molecular weight protein immunologically cross reacts with corn trypsin inhibitor.

Further work is required in order to understand the relationship between corn trypsin inhibitor and other spots which cross react with antibodies.

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Fig. 7. Alignment of corn trypsin inhibitor from cDNA clones and from protein sequence. cDNA sequence was adapted from Wen et al. (1988, personal communication). Protein sequence was adapted from Mahoney et al. (1984).

```
                1                   10                  20
CTI (cDNA):     S A G T S C V P G W A I P H N P L P S C R W Y V T
CTI (protein):  S A G T S C V P G W A I P H N P L P S C C W Y V T
                * * * * * * * * * * * * * * * * * * * *  * * * *

               26                  35                  45
CTI (cDNA):     S R T C G I G - - P R L P W P E L K R R C C R E L
CTI (protein):  S R R C G I G P R P R L P W P E L K R R C C R E L
                * *  * * * *    * * * * * * * * * * * * * * * *

               51                  60                  70
CTI (cDNA):     A D I P A Y C R C T A L S I L M D G A I P P G P D
CTI (protein):  A D I P A Y C R C T A L S I L M D G A I P P G P D
                * * * * * * * * * * * * * * * * * * * * * * * * *

               76                  85                  95
CTI (cDNA):     A Q L E G R L E D L P G C P R E V Q R G F A A T L
CTI (protein):  A Q L E G A L E D L P G C P R A V Q Q G F A A T L
                * * * * *  * * * * * * * *  * *  * * * * * * *

              101                 110                 120
CTI (cDNA):     V T E A E C N L A T I S G V A E C P W I L G G G T
CTI (protein):  V T E A E C N L E T I S - - - - - - - - - - - - -
                * * * * * * * *  * * *

              126
CTI (cDNA):     M P S K >
CTI (protein):  - - - - >
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Fig. 8. Reverse-phase HPLC profile of corn acidified extract. The salt extract corn protein was boiled and acidified as described in Methods. The sample was applied into the Synchroprep-RPP column (250x10 mm). The column was developed with a linear gradient from 0-60% acetonitrile contained 0.1% trifluoroacetic acid over 1 hour, at flow rate 3 ml/min. Inset: Dot blotting analysis of corn extract fractions collected from reversed-phase HPLC. Fractions were collected every two minutes started from 20 minutes, dried, then blotted onto nitrocellulose paper. The membrane was assayed as described in Methods.

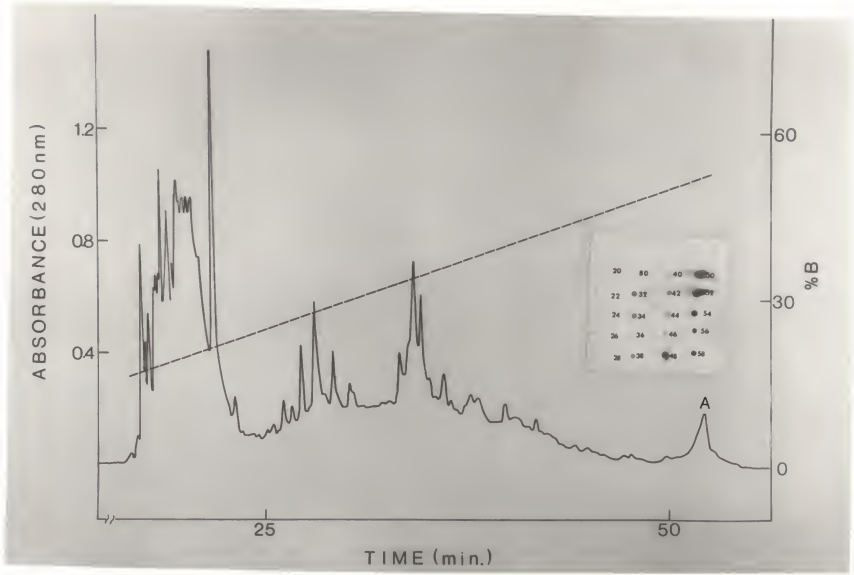

ABSORBANCE (280 nm)
1.2
0.8
0.4
60
30
0
%B
A
25
50
TIME (min.)

Fig. 9. Western blotting of corn extract fraction around peak A at Fig. 7. Lane 1-6 are fraction 48, 50, 52, 54, 56, 58 min. Lane 7, corn trypsin inhibitor purified according to Lei and Reeck (1986).

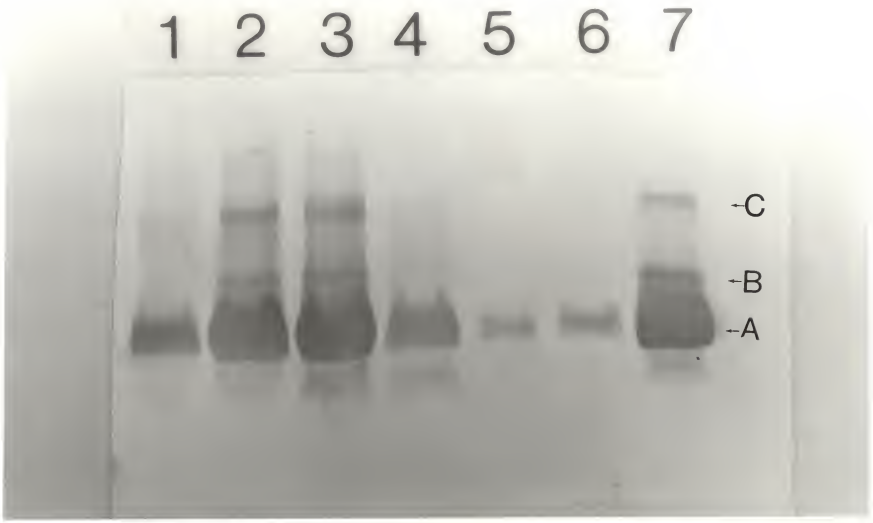

1 2 3 4 5 6 7
C
B
A

Fig. 10. Reversed-phase HPLC purification of corn trypsin inhibitor. Inject volume, 10 ml. Column was equilibrated at 30% acetonitrile containing 0.1% TFA. After flow through peak emerged, The gradient was increased up to 60% in 20 minutes, at Flow rate 3 ml/min.

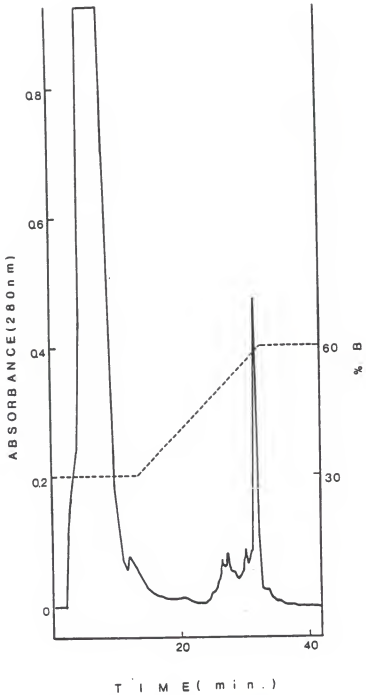

0.8
0.6
0.4
0.2
0
ABSORBANCE(280nm)
60
30
% B
20
40
TIME(min.)

Fig. 11. Reversed-phase HPLC purification of corn trypsin inhibitor. Sample was repeated injected into column five times (50 ml). Solvent system was the same as in Fig. 10.

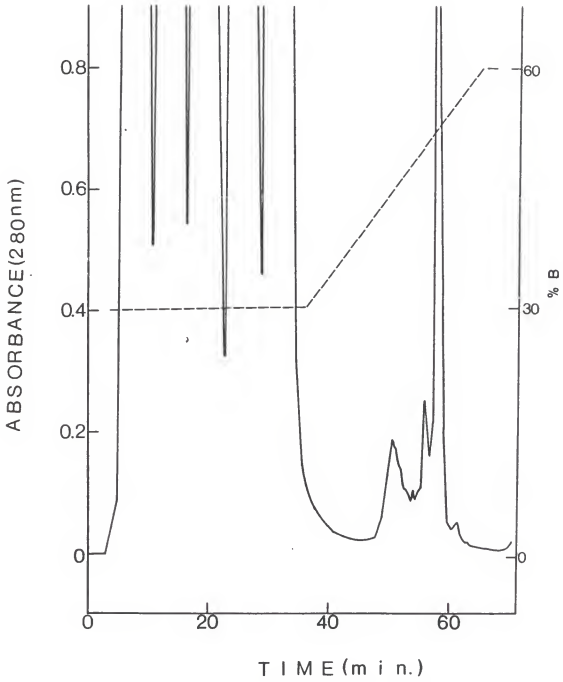

ABSORBANCE(280nm)
0.8
0.6
0.4
0.2
0
60
30
0
% B
0
20
40
60
TIME(min.)

Fig. 12 Two-dimensional electrophoresis analysis of corn trypsin inhibitor. First dimension was isoelectric focusing. Second dimension was SDS-polyacrylamide gel electrophoresis. Ampholine pH range 3.5-10.


IEF
SDS
Acidic
Basic

Fig. 13 Two dimensional electrophoresis analysis of corn trypsin inhibitor. First dimension was isoelectic focusing. Second dimension was SDS-polyacrylamide gel. Ampholine pH range 4-7.

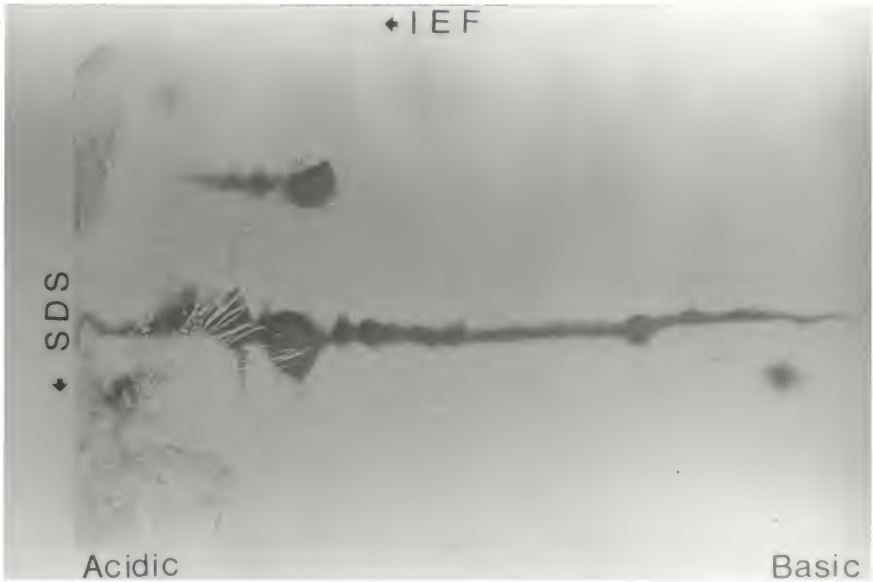
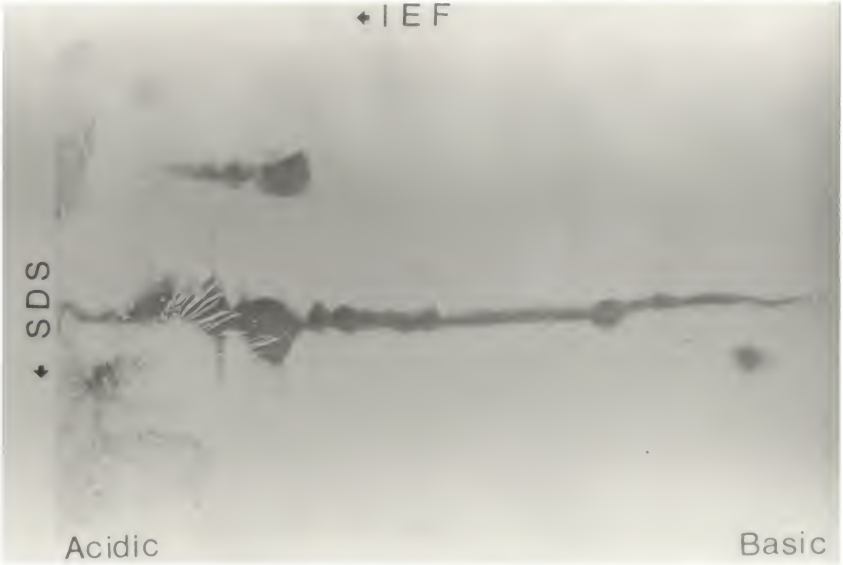

IEF
SDS
Acidic
Basic

Fig. 14 Two-dimensional Western blotting of corn trypsin inhibitor. The gel was run under the same condition as in Fig 11 and electroblotted onto nitrocellulose paper.


IEF
SDS
Acidic
Basic

ISOLATION AND CHARACTERIZATION OF CYANOGEN BROMIDE PEPTIDES OF RICE α-GLOBULIN AND PURIFICATION AND CHARACTERIZATION OF THE 12K CORN INHIBITOR

BY

KUO-CHANG ZEN

B. S. COLLEGE OF MARINE SCIENCE AND TECHNOLOGY,
TAIWAN, 1978.

AN ABSTRACT OF A MASTER'S THESIS
SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENT FOR THE DEGREE

MASTER OF SCIENCE
FOOD SCIENCE

KANSAS STATE UNIVERSITY
MANHATTAN, KANSAS

ABSTRACT

Rice α-globulin was isolated by repeated 30% ammonium sulfate and pH 4.5 precipitations. The protein was reduced, *S*-pyridylethylated, and digested by cyanogen bromide. The cyanogen bromide peptides were separated by reversed-phase and gel permeation high performance liquid chromatography. One of the peptides had a blocked N-terminus and was inferred to be the N-terminal of the whole protein. The C-terminal peptide, which contained no homoserine, was identified by amino acid analysis. Its complete (16-residue) sequence was determined, as was that as internal 10-residue peptide. The partial amino acid sequence of a large internal peptide indicated that α-globulin contains distinctive sequences with several glutamic acid/glutamine, serine or tyrosine residues in succession.

A single step purification of corn trypsin inhibitor was developed by using a semipreparative reversed-phase HPLC. The microheterogeneity of corn trypsin inhibitor was demonstrated in this study by two-dimensional gel electrophoresis and immunoassay.